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Long Term Potentiation (LTP) and Long Term Depression (LTD) Cause Differential Spatial Redistribution of the Synaptic Vesicle Protein Synaptophysin in the Middle Molecular Layer of the Dentate Gyrus in Rat Hippocampus

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Abstract. The presynaptic modifications that accompany long-term changes in synaptic plasticity are still not fully understood. Synaptophysin is a major synaptic vesicle protein involved in neurotransmitter release. We have used quantitative electron microscopy to study synaptophysin (Syn) immunolabelling in the hippocampus of adult rats 24h after induction *in vivo* of long term potentiation (LTP), and long term depression (LTD). Electrodes were implanted chronically in hippocampus with stimulation at either the medial (MPP) or lateral perforant path (LPP). 24h following induction of LTP or LTD rats were rapidly perfusion fixed and hippocampal tissue processed to electron microscopy via freeze substitution method. Anti-synaptophysin post-embedding immunolabelling was performed and tissue was imaged in the middle molecular layer (MML) of the dentate gyrus. There was a significant decrease in number of Syn labelled vesicles per unit area of bouton after LTP, but not LTD. An analysis of the spatial distribution of Syn labelled synaptic vesicles showed an increase in nearest neighbour distances, more so in the LTP than the LTD group, which is consistent with the overall decrease of Syn after LTP. These data are in agreement with the suggestion that Syn is involved in clathrin-dependent and "kiss and run" endocytosis which occurs perisynaptically. Thus, an increase in release of neurotransmitter and in consequence endocytosis would be consistent with an increased active zone distance for vesicles containing Syn.

Keywords: Synaptophysin, LTP, LTD, Dentate gyrus, Hippocampus, Electron microscopy

Synaptophysin is a synaptic vesicle glycoprotein with four transmembrane domains and has a molecular weight of 38 kD. It accounts for 7% of the total vesicle membrane proteins (Nadol et al., 1993) and is considered a reliable marker of nerve terminal differentiation due to its differential expression in terms of level and localization of immunoreactivity (Lupa and Hall, 1989). It has been identified as a calcium-binding protein (Shin, 2014) and hence may be a major component of the mechanism of neurotransmitter release (Reichardt et al., 1983). Synaptophysin has been considered as a molecular marker for the membrane of presynaptic vesicles, as well as a functional marker for synapses, and thus has been widely used to investigate synaptogenesis and synaptic loss in both animal models and human patients (Masliah et al., 1990, Kamikubo et al., 2006, Ojo et al., 2012; Wang et al., (2016). It has been also ben used as a marker synaptic number changes in the medial prefrontal cortex across adolescence in male and female rats (Dzewliecki et al., 2016). Li et al., (2002) found an increase in synaptophysin immunoreactivity 4 weeks after kindling stimulation in the stratum radium of CA1, stratum lucidum/radiatum of CA3, the hilus, the inner molecular layer of dentate gyrus and layer II/III of the piriform cortex of rat. An increase in the level of synaptophysin was observed in the hippocampus of environmentally enriched rats (Nithianantharajah et al., 2004). However, synaptophysin levels can also decrease in certain situations, e.g. in correlation with a loss of synapses in ageing, there is reduction in synaptophysin-immunoreactivity within hippocampal subfields (Davies et al., 2003, Ojo et al., 2012).

Two forms of activity-dependent synaptic plasticity, long-term potentiation (LTP) and depression (LTD) at glutamatergic synapses are thought to be involved in aspects of learning and memory (Bliss and Collingridge 1993; Linden and Conner 1995). In the dentate gyrus of the hippocampus, brief high-frequency afferent activity leads to a long-lasting increase in the strength of synaptic transmission (i.e. LTP) of the activated synapses, but a persistent reduction in synaptic strength (i.e. LTD) of non-activated synapses at converging inputs. More specifically, LTP and LTD in the middle molecular layer (MML) of the dentate gyrus in rat hippocampus occurs with, respectively, medial and lateral perforant paths high-frequency stimulation. Contrasting synaptic morphometry changes have been recorded after induction of LTP and LTD in the dentate gyrus of awake

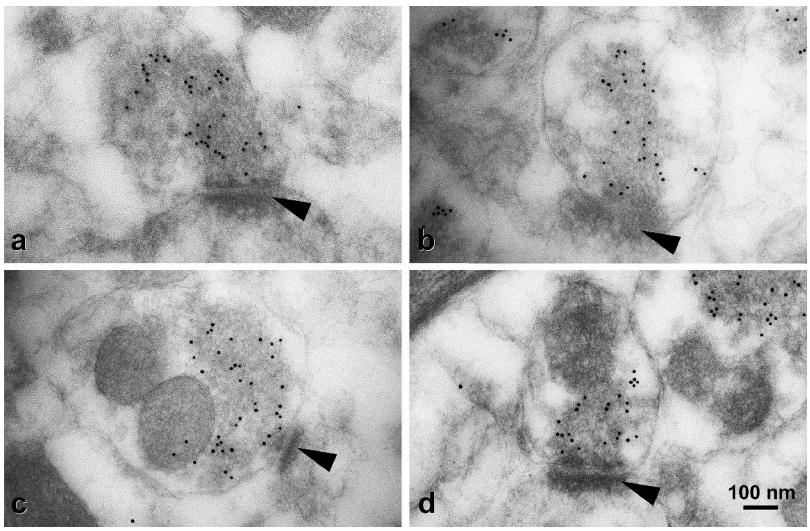

Figure 1 a-d. *Electron micrograph shows synaptophysin immunogold labelling in presynaptic vesicles in the middle molecular layer of the dentate gyrus in rat hippocampus. Arrowheads show the postsynaptic density in the active zone of the synapse. Scale bar 100nm.*

rats. However, these changes are not simply mirror phenomena (Mezey et al., 2004).

The changes in synaptic proteins and components following plasticity induced by phenomena such as LTP have been the subject of intensive investigation. Both hippocampal LTP (Antonova et al., 2001) and homosynaptic potentiation in Aplysia involve rapid recruitment of the presynaptic vesicle-associated protein synaptophysin (Jin et al., 2011). Also Huntley et al., (2012) using LTP, showed on light microscopy level that in potentiated MML, there was no change in density or size of synaptophysin puncta. However, the evidence for synaptophysin changes in LTD is less clear. Kamikubo et al., (2006) suggested that long-term depression (LTD) triggered a long-lasting reduction in synaptic strength accompanied by synapse elimination, and synaptophysin immunolabelling showed a significant decrease 3 weeks after the repeated application of LTD-inducing drugs including 3,5-dihydroxyphenylglycine (DHPG), but not after single treatments of these compounds.

In the present study we have used a quantitative immunocytochemical study at ultrastructural level to investigate the expression and distribution of synaptophysin immunostaining in synapses in the middle molecular layer of the dentate gyrus in rat hippocampus 24 hours after LTP, or LTD induction.

Materials and Methods

Animals

Nine adult male Sprague-Dawley rats (Iffa Credo, France) were used (weight ranging from 300–350g) at the time of surgery. They were housed individually with food and water ad libitum in a temperature-controlled room and on a 12-h light/dark cycle. All animal experimental procedures were carried out at Neurobiologie de l'Apprentissage, de la Mémoire et de la Communication, CNRS-Université Paris-Sud (now Paris-Saclay Institute of Neuroscience, Neuro-PSI), in accordance with the European Community Council Directive of 24 November 1986 and the guidelines of CNRS and the French Agricultural and Forestry Ministry (decree 87848; licence no. A91429).

Surgery and Induction of Long-Term Potentiation/ Long-Term Depression

The protocol for surgery and induction of long-term potentiation/long-term depression was same as in Mezey et al., 2004, and as prepared in the studies of Medvedev et al., 2010a,b. The tissue from middle molecular layer of the dentate was taken from animals 24h after either: (1) LTP (medial stimulation of perforant path, n=3), (2) LTD (lateral stimulation of perforant path, n=3) and (3) Control (no tetanic stimulation, n=3).

Fixation and Microscopy

Immediately after the last test stimulation, rats were anaesthetized with sodium pentobarbital (0.1mL/100g body weight) and perfused transcardially with 200ml of 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1M phosphate buffer. Brains were removed, the hippocampus dissected out and slabs of the dorsal part from the left hemisphere were soaked in 0.125M triethanolamine hydrochloride in 0.1M phosphate buffer at RT for 1 hour and cryo-protected using increasing concentrations of 10% to 30% glycerol in 0.1M phosphate buffer. The tissue was then frozen on a copper mirror cooled to -197oC with liquid nitrogen using a Leica MM80E, freeze-substituted at -85oC, infiltrated with Lowicryl HM20 at -50oC, embedded and polymerised in the Leica AFS under 360nm UV light for 48h at -50oC. Tissue samples were coded and all further procedures and analyses were performed 'blind'. All tissue analysed was at least 1 mm distant from electrode tracts in approximately the middle part of dorsal hippocampus.

For electron microscopy, ultrathin sections of silver–gold interference colour (~70nm) were cut from the dentate gyrus, including the entire molecular

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and granule cell layer, collected on pioloform/carbon coated nickel slot grids (2×1mm; Agar Scientific, UK). For post-embedding immunolabelling, sections were first incubated in 0.05M glycine in phosphate buffer for 20 minutes and then blocking buffer of PBS with 5% bovine serum albumin (BSA) and 0.1% cold-water fish gelatin (CWFG) for 15 minutes followed by incubation with mouse IgG anti-synaptophysin at 1:500 (Chemicon MAB 5258) in PBS with 0.8% BSA and 0.1% CWFG overnight at room temperature. After washing the sections were incubated in 10 nm gold-conjugated goat anti-mouse IgG at 1:50 (British BioCell), fixed in 2% glutaraldehyde and then counter-stained with aqueous 5% uranyl acetate. To check the specificity of the reaction, the primary antibody was excluded from a control set of sections. Finally, the sections were examined in an electron microscope (JEOL JEM 1010) at an accelerating voltage of 80kV and digital images were acquired using an AMT XR40 camera of 4 megapixels. Images from the middle molecular layer (MML) of the dentate gyrus were taken from halfway between the granule cell layer and the hippocampal fissure. All images were taken at 25000× magnification covering area of ~450μm2 in total for each animal.

Image Analysis

An analysis was made of immunolabelling of synaptophysin in presynaptic vesicles. For quantification, Image J software was used to measure the area of the synaptic boutons, and the number of gold particles/μm2 of bouton was counted in each of control, LTP and LTD treated rats. In addition the spatial distribution of synaptophysin labelled synaptic vesicles in MML of the DG of each of these 3 groups was measured using Neurolucida version 9 (MBF Bioscience, Williston, VT USA). For each labelled vesicle the nearest neighbour distance (NND) was plotted as a function of the distance to boutons active zone (AZD) (Control, N(boutons) = 283, N(particles) = 4190; LTP, N(boutons) = 277, N(particles) = 3331; LTD, N(boutons) = 249, N(particles) = 3376). These were expressed in 2-D histograms and a line graph for control, LTP and LTD rats in 50nm "bin" intervals up to a distance of 450nm from the boutons active zone. The mean NND and minimal AZD in boutons for all groups was plotted as well as the percentage of gold particles in three distinct pools (as described by Miranda et al., (2011)) depending on distance from the synapse active zone — docked pool (0 to 50nm from the active zone), the reserve pool (50 - 300nm) and the resting pool (>300nm).

Statistics

For statistical analysis the Microsoft Excel program was used. To examine differences between groups one-way ANOVA was performed followed by post hoc comparisons, where required, using the Bonferroni and Holm multiple comparison and planned comparison tests. Formal statistical significance was taken where $p<0.05$. Data are presented as mean ± SEM, n = 3 animals per group.

Results

The middle molecular layer (MML) of the dentate gyrus was identified as the area halfway between the cell body layer of the upper arm of the dentate gyrus and the presence of the hippocampal fissure. Immuno labelling of synaptophysin was specifically present in the presynaptic vesicles boutons (Fig 1 a-d). Control labelling was negative, as shown in our previous publication (Davies et al., 2003). The mean area of synaptic boutons measured in control, medial stimulation (LTP) and lateral stimulation (LTD) in MML was 0.12±0.03 μm2, 0.16±0.05 μm2 and 0.14±0.05 μm2, respectively. None of these differences were significantly different ($F2,6=0.684$, $p=0.54$) although.

Analysis of synaptophysin (Syn) immunolabelling in boutons did not show any differences between groups (Figure 2A, $F2,6=1.095$, $p=0.39$) when expressed as number

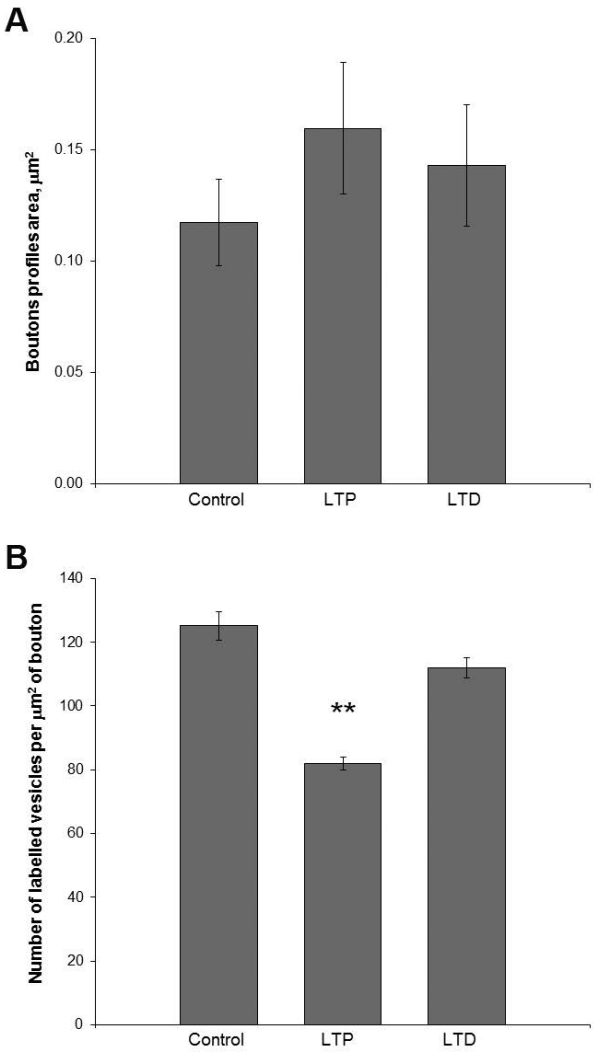

Figure 2 (A). *The number synaptophysin immuno labelled vesicles as identified by gold particles per bouton in the middle molecular layer of the dentate gyrus in Control, LTP and LTD rats and (B) the number of gold particles/μm2 of each bouton. The number of Syn labelled vesicles in LTP treatment is lower than in both LTD and control animals, ** p< 0.01 for LTP vs control and LTP vs LTD*

of labelled vesicles per bouton; however, when Syn immunolabelling was expressed as the number of labelled vesicles per μm2 of bouton area (Figure 2B), a significant decrease was noted in number of Syn labelled vesicles after LTP, but not after LTD ($F_{2,6}$=43.519, p=0.0003). An analysis of nearest neighbour distances between vesicles (NND) in 50nm bins up to 900 nm from the bouton active zone showed a more complex vesicle spatial arrangement (Figure 3A): and when this was plotted as means for each of control, LTP and LTD in the 50nm bins to 450 nm from the active zone it was apparent that there was significant increase in NND of Syn labelling in both the LTP and LTD animals compared to control (Figure 3B, post-ANOVA planned contrasts analysis t(30)=2.24, p=0.033), which is consistent with the overall decrease of Syn after LTP. Interestingly analysis of minimal distance from Syn labelled vesicles to boutons active zone (Figure 4A) showed a significant increase in minimal distance for the LTP group ($F_{2,6}$=5.231, p=0.048). The percentage of Syn-labelled vesicles in three pools in boutons was examined, namely the docked pool (0 to 50nm from the active zone), the reserve pool (50 - 300nm) and the resting pool (>300nm) (Figure 4B). There was a significant decrease in Syn in the vesicles of the docked pool of LTD synapses ($F_{2,6}$=5.698, p=0.041), but no significant differences in the reserved (50-300nm) or resting pools (>300nm from the active zone). We did not observe any qualitative difference in syn vesicle sizes in the docked compared to the reserved or resting zones.

Discussion

In a previous study on synaptic morphometry changes after LTP and LTD using unbiased counting methods (Mezey et al., 2004) it was shown that LTP and LTD were not in morphological terms simply mirror image phenomena. More recently, in an electron microscopy study involving detailed 3-D reconstructions of synapses and spine after LTP and LTD using the same parameters of induction as in the present study (Medvedev et al., 2010a), we showed that while there were no differences in synaptic density 24h after LTP or LTD induction, there were differences in the proportions of other synaptic parameters including dendritic spines. While LTP increased significantly

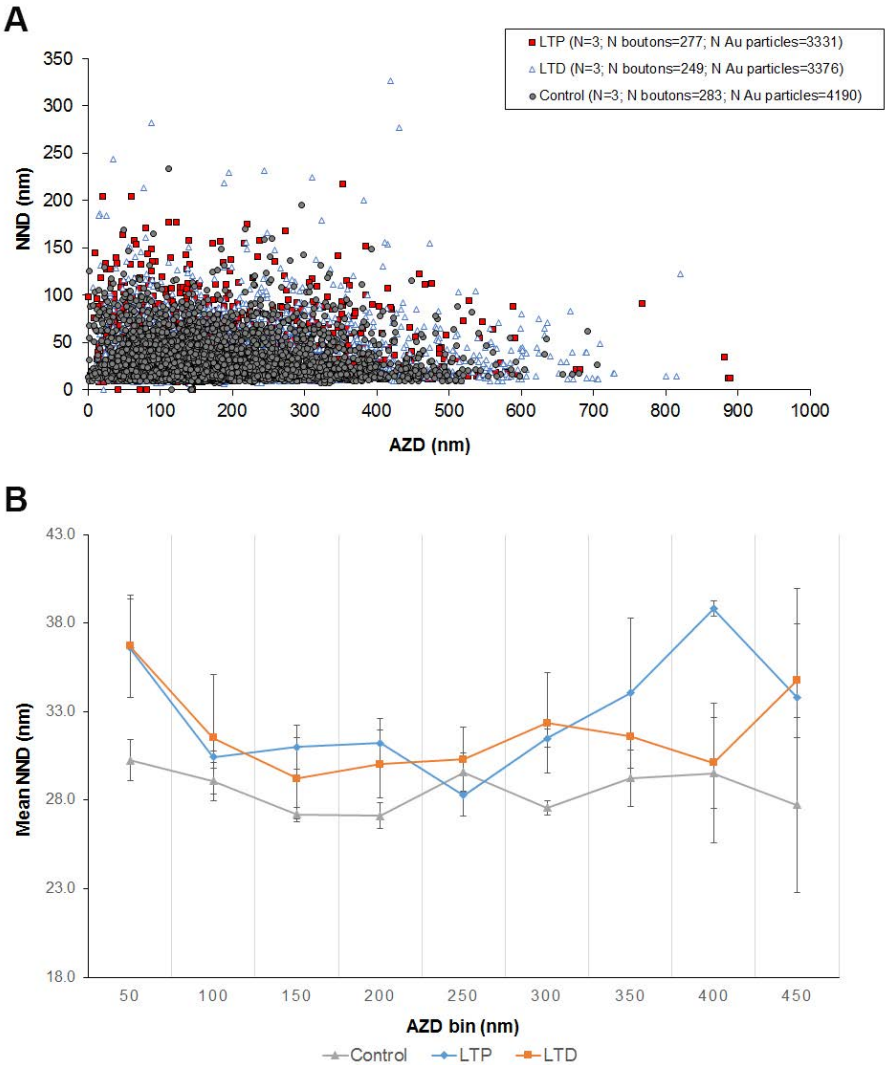

Figure 3. *NND of Syn labeled vesicles in control, LTP and LTD tissue. (A) For each labelled vesicle NND was plotted as a function of the distance to boutons active zone (AZD) (B) NND plotted as means for each of control, LTP and LTD in the 50nm bins to 450 nm from the active zone. There was significant increase in NND of Syn labelling in both the LTP and LTD animals compared to control (post-ANOVA planned contrasts analysis t(30)=2.24, p=0.033), which is consistent with the overall decrease of Syn after LTP.*

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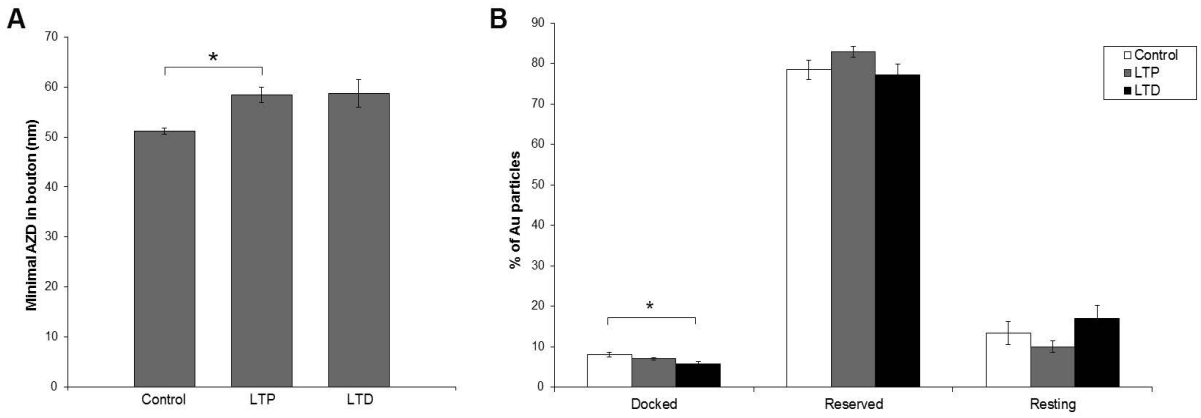

Figure 4 (A). *Minimal distance from Synaptophysin labelled vesicles to synaptic active zone in each bouton. There was a significant increase in minimal distance for the LTP group (F2,6=5.231, p=0.048) (B) Synaptophysin labelled vesicles in 3 defined pools in relation to the synaptic active zone: (i) docked – 0-50nm); (ii) reserved (50-300nm) and (iii) resting (>300nm). There was a significant decrease in Syn in the vesicles of the docked pool of LTD synapses (F2,6=5.698, p=0.041).*

the proportion of mushroom spines, LTD increased the proportion of thin spines, and both LTP and LTD decreased stubby spine number. Notably the volumes of pre-synaptic boutons on mushroom spines were smaller after LTD, indicating a pre-synaptic effect.

Our previous studies did not examine synaptic bouton vesicles, and our data here clearly show differences not only in the density of Syn-labelled vesicles per unit area of bouton (lower following LTP), but also differences in the spatial distribution of the vesicles in relation to the synaptic zone with a significant difference between LTP and control, which is consistent with the overall decrease of Syn following LTP, but also between LTD and control. The significance of these findings may have an explanation in relation to the process of fusion of vesicles to the active zone following LTP or LTD. The plasticity of synaptic connections depends on signal-processing specializations at synaptic-membrane active zones, which may control the functional and structural remodeling of synaptic connections (Olsen et al., 2006; Miranda et al., 2011) and our data may reflect a reduction of the readily-releasable pool of vesicles that could impact on the dynamics of replenishment processes, which is notable here during LTD.

Synaptophysin is a synaptic vesicle protein implicated in both exocytosis and endocytosis (Vatorta et al., 2004, Granseth et al., 2007). As Syn can regulate the fusion of the snare complex assembly through sequestration of the VAMP2 (Bacci et al., 2001), downregulation of synaptophysin would increase mEPSC frequency. Our data are in agreement with the suggestion that Syn is involved in clathrin-dependent and "kiss and run" endocytosis (Granseth, 2007) which occurs perisynaptically. Thus, an increase in release of neurotransmitter and in consequence endocytosis would be consistent with the significant increase in the distance from the boutons active zone of vesicles containing Syn.

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